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# Antimicrobial activity of *Callistemon rigidus* R.Br. leaf extract against multidrug resistant gram-negative clinical isolates

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**ABSTRACT**:
Infections due to gram-negative bacilli resistant to an array of structurally diverse antibiotics pose a therapeutic challenge and demand the discovery of alternative antimicrobial therapy. A lead fraction from *Callistemon rigidus* R.Br. leaves revealed potential bactericidal activity against multidrug resistant clinical isolates of *Escherichia coli* and *Pseudomonas* species. Agar well diffusion studies suggested a significant concentration dependent effect ($P < 0.0001$) of the test fraction against the isolates. Furthermore, minimum inhibitory concentration evaluated by *in vitro* microbroth dilution assay ranged between 0.625- 40µg/ml in contrast with the positive controls, cefepime and cefuroxime sodium exhibiting a minimum inhibitory concentration range of 0.625- 80µg/ml and 5-320µg/ml respectively.

**Key words**: Bactericidal activity, Multidrug resistant, *Escherichia coli*, *Pseudomonas*, *Callistemon rigidus* R.Br, Agar well diffusion, Microbroth dilution assay.

## INTRODUCTION

Gram-negative bacteria remain a major cause of community acquired and nosocomial infections. Several reports have documented the emergence of resistant variants especially among *Escherichia coli* and *Pseudomonas* species [1]. Increased availability and misuse of beta-lactam agents especially second and third generation cephalosporins has led to an explosive development and spread of extended spectrum beta-lactamase producing strains [2], thus limiting the treatment options and posing an enormous challenge to medicine and public health. Preserving the effectiveness of the existing therapies and discovery of newer, long-lasting therapies is thus essential. Further the discovery and development of cheap, readily accessible therapies to overcome intractable infections are the need of the hour.

Biomatrices like microbes, plants, animals are widely being explored from different geographic realm of the earth to develop new armamentarium against MDR pathogens. Plants are the oldest source of pharmacologically active compounds and have provided mankind with many useful medicines for years [3]. Biologically active plant extracts, pure compounds and their semi- synthetic derivatives serve as a promising therapeutic alternative to the currently available cost intensive options for the treatment of infections due to the superbugs [4].

*Callistemon rigidus* R.Br. (Myrtaceae) commonly known as bottlebrush is a stiff upright shrub characterized by red flower spikes that are shaped like bottlebrushes. The species is indigenous to tropics and Australia. Phytochemical investigation reveals aromatic nature of the plant owing to the higher content of α-pinene, 1,8 cineol and α-terpineol [5]. Traditional medicinal systems advocate different medicinal properties of the plant. Leaves have been used to cure cough, bronchitis and other respiratory tract infections in Cameroon, Australia, China and Asia [5].

However, experimental studies are lacking on the plant confirming its use in traditional medicinal systems to cure infections. It was found that *C. rigidus* leaves did not decay in the flower vase and hence was thought to possess some antibacterial principles (personal observation). Therefore based on the implicated ethnopharmacological use and personal observation the present study was designed to validate the *in vitro* antibacterial potential of the methanolic fraction of leaves of *Callistemon rigidus* against a panel of forty clinical isolates grouped as vancomycin- resistant *E. coli* (VRE), extended spectrum of β-lactamase producing *E. coli* (ESBL) and multi antibiotic resistant *Pseudomonas.*

## MATERIALS AND METHODS

### *Extraction:*

Leaves of *Callistemon rigidus* were collected in October 2004 from Thapar Technology Campus. Healthiness of the leaves was confirmed by culturing the cut sections of the leaves on nutrient agar and potato dextrose agar plates. Fresh and healthy leaves were thoroughly washed under running water for 30 minutes. The fresh weight of the leaves was recorded and these were then subjected to drying at 35˚C and ground to a fine powder. The pulverized plant material (70g) was extracted exhaustively by soxhlet at 50°C using methanol (150ml) as the solvent. The solvent was filtered and the filtrate evaporated to obtain a brown residue referred to as the crude extract. The weight of the crude extract was recorded to calculate the % yield.

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**Table 1: Culture collection**

| Species | Culture ID | Source | Repository |
|---|---|---|---|
| **PUS CULTURES** | | | |
| *Escherichia coli* | Eco (A)1 | Pus | AIIMS |
| *Escherichia coli* | Eco G1 | Pus | GMCP |
| *Escherichia coli* | Eco G2 | Pus | GMCP |
| *Escherichia coli* | Eco G3 | Pus | GMCP |
| *Pseudomonas aeruginosa* | Pae G1 | Pus culture | GMCP |
| *Pseudomonas aeruginosa* | Pae G2 | Pus for C/S | GMCP |
| *Pseudomonas aeruginosa* | Pae G3 | Pus for C/S | GMCP |
| *Pseudomonas aeruginosa* | Pae G4 | Pus for C/S | GMCP |
| *Pseudomonas sp.* | P(G)1 | Pus for C/S | GMCP |
| **URINE CULTURES** | | | |
| *Escherichia coli* | Eco (A)2 | Urine culture | AIIMS |
| *Escherichia coli* | Eco (A)3 | Urine culture | AIIMS |
| *Escherichia coli* | Eco G4 | Urine C/S | GMCP |
| *Escherichia coli* | Eco G5 | Urine C/S | GMCP |
| *Escherichia coli* | Eco G6 | Urine C/S Prostractomy | GMCP |
| *Escherichia coli* | Eco G7 | Urine C/S | GMCP |
| *Escherichia coli* | Eco G8 | Urine C/S | GMCP |
| *Pseudomonas aeruginosa* | Pae G5 | Urine C/S | GMCP |
| *Pseudomonas aeruginosa* | Pae G6 | Urine C/S | GMCP |
| *Pseudomonas sp.* | P(A)3 | Urine C/S | AIIMS |
| **BLOOD CULTURES** | | | |
| *Pseudomonas sp.* | P(A)1 | Drain culture | AIIMS |
| *Pseudomonas aerogenes* | Par (A)1 | Endocarditis | AIIMS |
| *Pseudomonas aerogenes* | Par(A)2 | Endocarditis | AIIMS |
| *Pseudomonas sp.* | P(G) 2 | Blood culture | GMCP |
| *Escherichia coli* | Eco(A)4 | Blood culture | AIIMS |
| **VAGINAL SWABS** | | | |
| *Escherichia coli* | Eco G9 | Vaginal swab C/S | GMCP |
| *Escherichia coli* | Eco G10 | Vaginal swab C/S | GMCP |
| *Escherichia coli* | Eco G11 | Vaginal swab C/S | GMCP |
| *Escherichia coli* | Eco G12 | Vaginal swab C/S | GMCP |
| *Escherichia coli* | Eco G17 | Vaginal swab C/S | GMCP |
| **GASTROINTESTINAL PATHOGENS** | | | |
| *Escherichia coli* | Eco NDRI | | NDRI, Karnal |
| *Escherichia coli O157:H7* | Eco G14 | | |
| *Escherichia coli O157:H7* | Eco G15 | | |
| **TRACHEAL CULTURES** | | | |
| *Escherichia coli* | Eco G13 | Endotracheal tube secretion | GMCP |
| *Pseudomonas sp.* | P(A)2 | Tracheal Culture | AIIMS |
| **BURN SWABS** | | | |
| *Pseudomonas aeruginosa* | Pae G7 | Pus for C/S | GMCP |
| **EAR DISCHARGE SWABS** | | | |
| *Pseudomonas aeruginosa* | Pae G8 | Ear discharge | GMCP |
| *Pseudomonas aeruginosa* | Pae G11 | Ear discharge swab | GMCP |
| **OTHER CULTURES** | | | |
| *Escherichia coli* | Eco (LHMC) | Antibiotic sensitivity testing | LHMC |
| *Escherichia coli* | Eco G16 | Urinary pathogen | GMCP |
| *Escherichia coli* | Eco(A)5 | Resistant to all antibiotics | AIIMS |

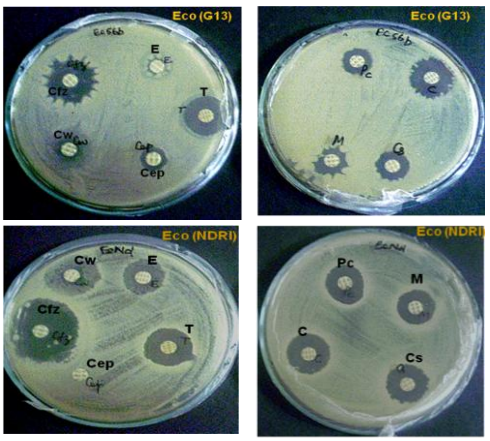
AIIMS: All India Institute of Medical Sciences, New Delhi, INDIA; GMCP: Government Medical College, Patiala, INDIA: NDRI; National Dairy Research Institute, Karnal, INDIA; LHMC: Lady Harding Medical College, New Delhi, INDIA.

The crude extract was partitioned between chloroform and methanol (1:1) in a separating funnel using the technique of liquid-liquid fractionation. Chloroform and methanol fractions were pooled separately and evaporated to obtain the respective residues. The residues were stored at -20˚C until further use.

***Test microorganisms:*** The test microorganisms used included multidrug resistant clinical isolates of E. coli (23 isolates) and Pseudomonas sp. (17 isolates), (Table 1). The cultures were procured from Department of Microbiology, All India Institute of Medical Sciences (AIIMS), New Delhi; Department of Microbiology, Government Medical College (GMC), Patiala; Lady Harding Medical College, New Delhi and National dairy Research Institute (NDRI), Karnal. The bacteria were maintained on trypticase soy agar slants and the cultures were activated in cation adjusted Mueller Hinton (MH) broth 18- 24 hours prior to the test. Susceptibility testing of all bacterial and fungal isolates was done by antibiogram evaluation using Kirby Bauer Disk Assay [6, 7], based on which the cultures were grouped as vancomycin resistant E. coli, extended spectrum β-lactamases producing E. coli and multidrug resistant Pseudomonas sp (Table 2, Fig. 1). All isolates were resistant to one or more antibiotics. *E.coli* NCTC 10418 was included as the reference strain.

**Table 2: Culture classification based on their susceptibility testing**

| Resistance | No. of isolates | Culture Id |
|---|---|---|
| Vancomycin resistant *E.coli* | 08 | Eco A3, Eco A4, Eco A5, Eco G2, Eco G3, Eco G4, Eco G6, Eco G7 |
| Extended spectrum beta lactamase producing *E.coli* (ESBL's) | 13 | Eco A1, Eco A2, Eco A4, Eco A5, Eco G1, Eco G2, Eco G3, Eco G4, Eco G7, Eco G15, Eco G16 and Ec Nd |
| Pae- *Pseudomonas aeruginosa*, Eco- *Escherichia coli*, Multiantibiotic resistant Pseudomonas (MARP) | 03 | Pae G4, Pae G8 and Pae G12 |




Codes of the antibiotic discs are as follows: *M-Methicillin; Pc- Piperacillin; E-Erythromycin; Cfz- Cefprozil; Cs-Cefoperazone; T-Tetracycline;Cep-Cefpodoxime*

**Fig 1: Antibiogram by Kirby Bauer Disk Assay**

***Susceptibility testing of test isolates:***

Antibiogram is the resistance pattern of a microorganism against a battery of antimicrobial agents. The Kirby Bauer disk assay was used for testing 38 antibacterial drugs against the test microorganisms for profiling their resistance pattern according to Clinical Laboratory Standards Institute (CLSI, USA, 1997) (formerly National Committee for Clinical Laboratory Standards -NCCLS) guidelines. The turbidity of activated culture of the test organism was visually adjusted using sterile saline solution (0.85% NaCl solution) to approximately that of 0.5 McFarland turbidity standard (1.5 x $10^8$ CFU/ml). Within 15 minutes of adjusting the inoculum to McFarland 0.5 turbidity standard, Muller Hinton agar plates (mean depth ± 4.00mm) were inoculated with the test culture by swabbing each plate 3 times for carrying out the antibacterial resistance profiles respectively. Sterile antibiotic discs (HiMedia) were applied to the agar surface using a sterile dispenser and applying gentle pressure with sterile forceps to ensure complete contact of disk with agar. Plates were incubated for 24 hours at 37°C in a BOD incubator. Susceptibility was evaluated by measuring the diameter of the clear inhibition zone across the antibiotic disk in lawn of microorganism. Plates were examined visually for isolated colonies within the inhibition zone that may have represented resistance. All the experiments were repeated three times. Based on the diameter of inhibition zone the cultures were classified as sensitive, intermediate or resistant.

***Determination of antibacterial activity:***

Sensitivity test for chloroform and methanol fraction residues was performed by agar well diffusion assay [8]. 5mm wells were cut in MH agar plates (mean depth ± 4.00mm). The extract was evaluated at six different concentrations (16.65µg, 33.3µg, 66.6µg, 99.9µg, 199.8µg and 399.9µg). 30µl of the test extract in Dimethylsulfoxide (DMSO) was dispensed in the test wells. Solvent blank was included as the control. The wells were sealed with molten MH agar. After 15 minute the plate was swabbed with 18-24 hours old 0.5 McFarland adjusted culture of the test isolate. Antibacterial activity was interpreted by determining the diameter of inhibition zone (DIZ) formed across the test well calculated by measuring the distance between the edge of the well and the outer edge of the inhibition zone.

***In vitro* determination of minimal inhibitory concentration:**

Agar well diffusion studies indicated the antimicrobial potential of methanolic fraction of the leaf extract of *Callistemon rigidus*. Based on this, only the methanolic leaf fraction residue was evaluated for the determination of Minimal Inhibitory Concentration (MIC) by microbroth dilution method using 96 well microtitre plate and 3-(4, 5-Dimethyl-2-thiazolyl -2, 5-diphenyl-2H)-tetrazolium bromide (MTT) assay. The assay was done using the standard testing methodology for evaluation of antimicrobial agents as recommended by EUCAST [9]. The range of antimicrobial dilutions evaluated was 320- 0.625 µg/mL. The turbidity of the test inoculum was adjusted visually by comparing it with 0.5 McFarland standard against a sheet of white paper on which sharp black lines were drawn. 50µl of the 0.5 McFarland adjusted bacterial suspension in saline was added to 125µl of MH broth to achieve a final bacterial cell concentration of $10^6$ cells in the well. Plates were then incubated at 37˚C for 2.5 hours after which 25µl of the test extract at different concentrations (0.625µg/mL; 1.25µg/mL; 2.5µg/mL; 5.0µg/mL; 10.0µg/mL; 20.0µg/mL; 40.0µg/mL; 80.0 µg/mL; 160.0µg/mL and 320.0µg/mL) was added. After 24 hours, 20µl of 0.02% MTT was added to each well. The assay was performed in triplicates. Cefepime and cefuroxime sodium, fourth and second generation cephalosporins respectively were included in the study as positive controls. Statistical analysis by two- way analysis of variance (2-way ANOVA) revealed significant effect ($P< 0.0001$) of the test parameters namely isolates type and concentration of extract on the inhibition zone [10, 11]. Pearson correlation is a very important parameter in understanding the dose-drug behavior as well as drug efficacy in terms of post antibiotic effect [12].

**RESULTS AND DISCUSSION**

Agar well diffusion studies demonstrated antibacterial activity of the methanol fraction residue (Table 3). The chloroform fraction did not show any antibacterial activity. There was a highly significant correlation between the dose of the extract and diameter of inhibition zone against both groups of test isolates. The $R^2$ value of drug concentration to inhibition zone diameter was 0.9745 in *Pseudomonas* species, and 0.9544 in *Escherichia* species (Fig.2 & 3)

Methanolic fraction residue was active against 82% of the test isolates within a MIC range of 0.625- 40µg/ml. Excluding Eco G3 and Eco G4, the fraction exhibited significant activity against vancomycin resistant *E. coli* and ESBL strains. All isolates of *Pseudomonas* except Pae G2, P A1 and Par A1 were susceptible to the test fraction at concentration ≤ 2.5µg/ml. Cefepime was active against 77% of the test isolates and had a MIC range of 0.625-80µg/ml. Cefuroxime sodium was active against only 47% of the isolates with a MIC range of 5-320µg/ml. $MIC_{90}$ of the methanol fraction residue was 10µg/ml as compared to cefepime having $MIC_{90}$ of 40µg/ml.

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**Table 3.** Comparative *in vitro* antimicrobial activity of methanolic fraction residue from leaves of *Callistemon rigidus*, cefepime and cefuroxime sodium by agar well diffusion and microbroth dilution assays against multidrug resistant *Escherichia coli* and *Pseudomonas*

| S.No. | Culture | DIZ at 199µg (dia in mm) DIZ± SE | Minimum Inhibitory Concentration (µg/ml) | | |
|---|---|---|---|---|---|
| | | | Methanolic | Cefepime | Cefuroxime sodium |
| 1 | Eco A1 | 140± 0.1 | 10 | 20 | No activity |
| 2 | Eco A2 | 24± 0.1 | 10 | 10 | No activity |
| 3 | Eco A3 | 22± 0.4 | 40 | No activity | No activity |
| 4 | Eco A4 | 20± 0.5 | 5 | 10 | No activity |
| 5 | Eco A5 | 16± 0.1 | 5 | 10 | No activity |
| 6 | Eco (LHMC) | 14± 0.1 | 2.5 | 10 | 10 |
| 7 | Eco G1 | 6± 0.0 | 1.25 | 5 | 80 |
| 8 | Eco G2 | 0± 0.0 | 0.625 | No activity | 5 |
| 9 | Eco G3 | 18± 0.5 | No activity | No activity | No activity |
| 10 | Eco G4 | 15± 0.7 | No activity | No activity | No activity |
| 11 | Eco G5 | 20± 0.2 | 5 | 160 | No activity |
| 12 | Eco G6 | 14±0.5 | 20 | 10 | No activity |
| 13 | Eco G7 | 15± 0.5 | 0.625 | No activity | No activity |
| 14 | Eco G8 | 16± 0.3 | 0.625 | 20 | No activity |
| 15 | Eco G9 | 0± 0.0 | 0.625 | 20 | No activity |
| 16 | Eco G10 | 8± 0 | 2.5 | 20 | 80 |
| 17 | Eco G11 | 8± 0 | No activity | No activity | No activity |
| 18 | Eco G12 | 13± 0.2 | No activity | 40 | No activity |
| 19 | Eco G13 | 15± 0.3 | 2.5 | 10 | No activity |
| 20 | Eco G14 | 18± 0.3 | 20 | 20 | No activity |
| 21 | Eco G15 | 15± 0.6 | 5 | 40 | No activity |
| 22 | Eco G16 | 14± 0.3 | 5 | 40 | No activity |
| 23 | Eco Nd | 25± 0.3 | 10 | 20 | 20 |
| 24 | PA1 | 17± 0.3 | 1.25 | 10 | 80 |
| 25 | P A2 | 18± 0.4 | 2.5 | 10 | 80 |
| 26 | P A3 | 15± 0.6 | 0.625 | 10 | 40 |
| 27 | Par A1 | 14± 0.5 | No activity | No activity | No activity |
| 28 | Par A2 | 19± 0 | 0.625 | 0.625 | 80 |
| 29 | Pae G1 | 0± 0 | 0.625 | 40 | 40 |
| 30 | Pae G2 | 7± 0 | No activity | No activity | No activity |
| 31 | Pae G3 | 11± 0 | 2.5 | 40 | 320 |
| 32 | Pae G4 | 9± 0 | 2.5 | 80 | 160 |
| 33 | Pae G5 | 16± 0.2 | 2.5 | 80 | 160 |
| 34 | Pae G6 | 30± 0.2 | 1.25 | 40 | 80 |
| 35 | Pae G7 | 18± 0.4 | 2.5 | 40 | No activity |
| 36 | Pae G8 | 14± 0.5 | 1.25 | 40 | 80 |
| 37 | Pae G12 | 15± 0.4 | 1.25 | 40 | 80 |
| 38 | Ps VP | 19± 0.5 | 1.25 | 40 | 80 |
| 39 | P(G)1 | 18± 0.1 | 2.5 | 40 | 80 |
| 40 | P(G)2 | 22± 0.5 | No activity | 40 | 80 |

*DIZ- diameter of inhibition zone; SE- Standard error*

Despite the availability of an array of clinically useful anti-infectives, the development of new antimicrobics, particularly against multidrug resistant pathogenic microorganisms is an important field of medical exploration. Due to the wide variety of structural diversity in phytochemicals [13], plants are a potential active source against superbugs. Different methods have been employed for revealing the anti-infective properties of plants. Agar well assay is a widely used assay. Antibacterial potential of numerous plants has been evaluated by agar well assay [14, 15]. The next standard used to evaluate the antibacterial potential in plants is *in vitro* evaluation of MIC [14, 16, 17, 18]. Studies using agar well diffusion assay have also revealed potential antibacterial activity of crude methanolic extract of leaves of *Callistemon rigidus* against both gram positive and gram negative human pathogenic isolates [19].

Methanolic extract of *Myrtus communis* also a Myrtaceae family member exhibits a MIC of 1mg/ml, which is very high compared to our extract [20]. Similarly, acetone extract from the bark of *Syzygium jambos* (Myrtaceae) exhibits a MIC of ≥ 1mg/ml against clinical isolates of *E. coli* [21]. Our study confirms the bioactive potential of methanolic fraction residue from the leaves of *Callistemon rigidus* and its possible use in the present armamentarium of antimicrobial therapy to combat the superbugs *Escherichia coli* and *Pseudomonas* species by isolation of the bioactive compound. Further studies for the purification and identification of the active antibacterial constituents of *Callistemon rigidus* are underway.

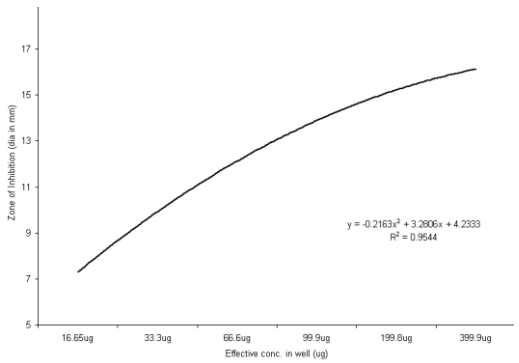


**Fig.2: Agar well diffusion assay of test extract against *Escherichia coli***

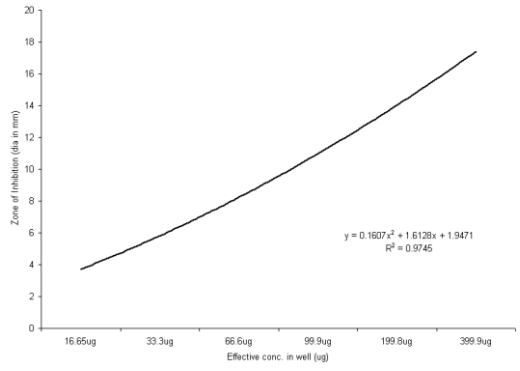


**Fig.3: Agar well diffusion assay of test extract against *Pseudomonas sps***

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